# Overview of Cell Culture Technology



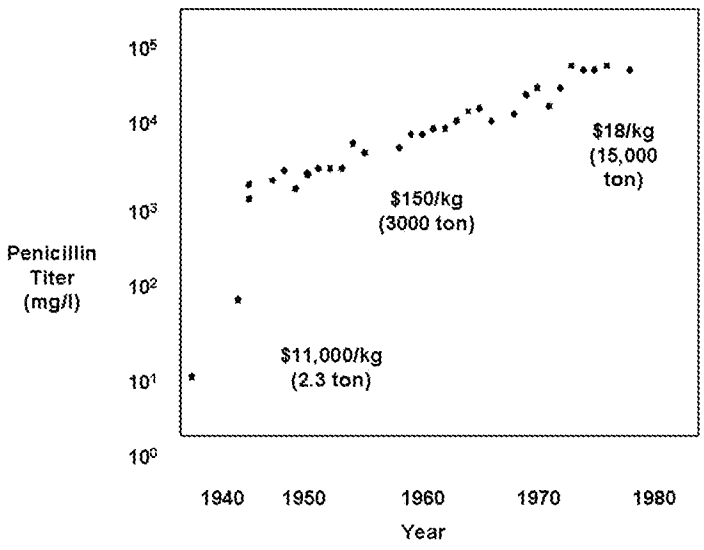
## Cell Culture Engineering - Where we have come from and where we are headed

In the past decade, we have seen continuing growth in mammalian cell culture bioprocessing, primarily driven by the expanding antibody production industry. This expansion is not only in the number of products now available to needy patients, but also in the quantity of antibody produced. Further raising the excitement level is the growing number of prospective therapeutic products for diseases that are likely to become treatable. Although it has been over two decades since the employment of mammalian cell culture in recombinant therapeutic production, and a decade since cell culture bioprocessing was proclaimed to be a "mature technology," the new surge in the quantities of products required and the phenomenally high investment cost for a manufacturing plant have spurred a new drive to enhance cell culture bioprocess technology. Recombinant therapeutic proteins have made a major headway in healthcare. Their future societal impacts may rival those of antibiotics, whose discovery and clinical applications have heralded the arrival of modern medicine. Like microbial fermentation technology in the 1950s, which allowed penicillin to become widely available, advances in cell culture processing technology have been instrumental in making these new medicines available to patients.

When assessing the biotechnological potential of process enhancement, it is instructive to look back at the history that preceded major biotechnological drugs.

**EXHIBIT O**

From its discovery by Sir Alexander Flemming and its expansion into clinical applications by Edward Penley Abraham until the end of its second decade, both the product titer and the production volume of penicillin increased almost exponentially. This was followed by a steady but slower enhancement in process, in terms of the product titer, in the following half a century. The roughly three orders of magnitude increase in production volume and product concentration was the result of decades of relentless effort by process scientists and engineers through strain improvement, media development and many other process innovations. Oxygen and heat transfer capacity and mixing characteristics have steadily pushed upward the performance level. On-line sensors, sterility control, equipment reliability and process control, all enhanced drastically over the past decades. However, the success of process technology eventually drove down the price. Penicillin G is no longer produced in the United States, as the cost of production is dramatically lower in some other parts of the world.




With some imagination, one may see a close resemblance of cell culture products to the historical graph of penicillin. Nearly two decades after their first introduction as therapeutic biologics, we have seen process technology make large manufacturing processes a reality, with a steady increase in titer, from tens of milligrams per liter in hybridoma cultures in the 1980s, to four or five grams per liter for some immunoglobulin products today. Although little published information is available, the production cost has also reduced by at least an order of magnitude since the beginning of cell culture products. If one were to plot such a graph for cell culture products for one or two decades from now, we are likely approaching the end of the rapid exponential stage in terms of product titer and process economics. However, in terms of real quantity, tremendous process enhancement was accomplished even after the initial rapid growth phase was over. The question for bioprocess scientists and engineers to ponder is what will it take for cell culture processing to accomplish what the antibiotics industry has achieved in our society in making major medicines affordable to the world's population.

What bioprocess scientists and engineers possess today that was not available to antibiotic researchers or even to the initial innovators in cell culture processes is the ready availability of genomic exploration and cell engineering tools. These new genome-wide investigative tools will greatly facilitate the discovery of genes crucial to conferring cells with desired growth and production characteristics. The new methodology of targeting

host genes for modulation of their expression level leading to altered cell physiology will result in greater productivity as well as increased robustness of the process. Will also facilitate the expansion of biosimilars (Follow-on) and make many medicines available to needy patients around the world at an affordable cost.

## Cell Culture Products and Manufacturing Products

**Principal Viral Vaccines Used in Prevention of Human Virus Diseases**

| Disease | Source of vaccine | Condition of virus |
|---|---|---|
| Poliomyelitis | Tissue culture (human diploid cell line, monkey kidney) | Live attenuated |
| Measles | Tissue culture (chicken embryo) | Live attenuated |
| Mumps | Tissue culture (chicken embryo) | Live attenuated |
| Rubella | Tissue culture (duck embryo, rabbit, or human diploid) | Live attenuated |
| Smallpox (vaccinia) | Lymph from calf or sheep (glycerolated, lyophilized) | Live vaccinia |
| Smallpox (vaccinia) | Chorioallantois, tissue cultures (lyophilized) | Vaccinia |
| Yellow fever | Tissue cultures and eggs (17D strain) | Live attenuated |
| Influenza | Highly purified subunit forms of chicken embryo allantoic fluid (formalinized UV irradiated) | Inactivated |
| Influenza | Cell culture (MDCK, Vero) | Attenuated |
| Rabies | Duck embryo or human diploid cells | Inactivated |
| Adenovirus | Human diploid cell cultures | Live attenuated |
| Japanese B encephalitis | Mouse brain (formalinized), cell culture | Inactivated |
| Venezuelan equine cephalomyelitis | Guinea pig heart cell culture | Live attenuated |
| Eastern equine encephalomyclitis | Chicken embryo cell culture | Inactivated |
| Western equine encephalomyelitis | Chicken embryo cell culture | Inactivated |
| Russian spring - summer encephalitis | Mouse brain (formalinized) | Inactivated |
| Hepatitis A | Cell Culture MRC-5 | Inactivated |
| Rotavirus | Multivalent bovine | Cell Culture |
| Varicella Zoster (Chicken Pox) | Cell Culture MRC-5 | Inactivated |

**Therapeutic Protein Biologics Produced in Non-Mammalian Host**

| | Activity/Use |
|---|---|
| Granulocyte colony-stimulating factor (Neupogen) | White blood cell growth for Neutropenia |
| Insulin (Humulin) | Diabetes |
| α-Interferon (Intron-A) | Anticancer, viral infections |
| Somatropin [human growth hormone] (Humatrope) | Growth deficiencies |
| Somatropin [human growth hormone] (Protopin/Nutropin) | Growth deficiencies |
| Interleukin-2 (Proleukin) | Kidney Cancer |

*Non-Antibody Product*

| Trade name | Type | Therapeutic Use | Manufacturer | U.S. approval year | Host |
|---|---|---|---|---|---|
| Aldurazyme | Laronidase | Mucopolysaccharid-eosis I | Genzyme | 2006 | CHO |
| Cerezyme | β-glucocerebrosidase | Gaucher's disease | Genzyme | 1994 | CHO |
| Myozyme | α-galactosidase | Pompe disease | Genzyme | 2006 | CHO |
| Fabrazyme | α-galactosidase | Fabry disease | Genzyme | 2003 | CHO |
| Naglazyme | N-acetylgalactosamie 4-sulfatase | Mucopolysaccharideosis VI | BioMarin | 2005 | CHO |
| Orencia | Ig-CTLA4 fusion | Rheumatoid arthritis | Bristol-Myers Squibb | 2005 | CHO |
| Luveris | Luteinizing hormone | Infertility | Serono | 2004 | CHO |
| Activase | Tissue plasminogen activator | Acute myocardial infraction | Genentech | 1987 | CHO |

**Therapeutic Antibody Products**

| Trade name | mAb type | Therapeutic Use | Manufacturer | U.S. approval year | Host |
|---|---|---|---|---|---|
| Orthoclone OKT3 | Muromomab CD3 | Reversal of acute kidney transplant rejection | Johnson & Johnson | 1986 | Hybridoma |
| ReoPro | Abciximab | Prevention of blood clots | Centocor | 1994 | SP2/0 |
| Rituxan | Anti-CD20 mAb | Non-Hodgkin's lymphoma | Genentech, Biogen IDEC | 1997 | CHO |
| Zenapax | Daclizumab | Prevention of acute kidney transplant rejection | Protein Design Labs | 1997 | NS0 |
| Simulect | Basiliximab | Prophylaxis of acute organ rejection in allogeneic renal transplantation | Novartis | 1998 | |
| Synagis | Palivizumab | Prophylaxis of lower-respiratory-tract disease | MedImmune | 1998 | CHO |
| Remicade | Anti-TNF-α-mAb | Active Crohn's disease | Centocor | 1998 | SP2/0 |
| Herceptin | Anti-HER2 mAb | Metastatic breast cancer | Genentech | 1998 | CHO |
| Mylotarg | Anti-CD33 | Acute myeloid leukemia | Wyeth | 2000 | CHO |
| Campath | Anti-CD52 mAb | Chronic lymphocytic leukemia | Millennium, Berlex, Genzyme | 2001 | CHO |
| Zevalin | Anti-CD20 murine mAb | non-Hodgkins lymphoma | Biogen IDEC | 2002 | CHO |
| Humira | Anti-TNF-α mAb | Rheumatoid arthritis | Abbott | 2002 | CHO |
| Xolair | Anti-IgE mAb | Moderate/severe asthema | Genentech | 2003 | CHO |
| BEXXAR | Anti- CD20 mAb | Follicular non-Hodgkins lymphoma | GSK | 2003 | CHO |
| Raptiva | Anti-CD11a mAb | Chronic psoriasis | Genentech | 2003 | CHO |
| Erbitux | Chimeric antibody raised against human EGF receptor | EGF receptor--expressing metastatic colorectal cancer | Imclone Systems, Bristol-Myers Squibb, Merck | 2004 | CHO |
| Avastin | Anti-VEGF | Metastatic colorectal cancer and lung cancer | Genetech | 2004 | CHO |
| Soliris | Antibody binding to C5 | Paroxysmal nocturnal hemoglobinuria | Alexion | 2007 | NS0 |
| Vectibix | Anti-EGFR mAb | Metastatic colorectal cancer | Amgen | 2006 | CHO |

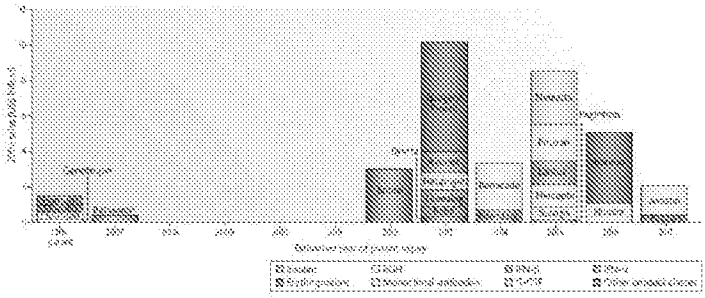
## Industrial Cell Lines

| **Major Cell Strains and Lines for Human Biologics Production** | |
|---|---|
| **Human Vaccines** | |
| Primary Cells | Green monkey kidney cells (no longer used) |
| | Chicken embryo cells |
| Cell strains | MRC5 (human lung fibroblast) |
| Cell line | Vero (monkey kidney epithelial cell) |
| **Recombinant Proteins** | |
| *Species cell line derived from* | |
| Human | HEK 293, Per C6 |
| Mouse | C-127, NSO, hybridoma cells, SP2/0 |
| Chinese Hamster | CHO |
| Syrian hamster | BHK |

## Cell Lines Used in the Production of Veterinarian Vaccines*

| **Vaccines** | **Cell line** |
|---|---|
| Bovine viral diarrhea virus | MDBK |
| Bovine parainfluenza virus type 3 | MDBK |
| Bovine rhinotracheitis virus | MDBK |
| Bovine respiratory syncytial virus | MDBK |
| Feline leukemia virus | FL72 |
| Feline panleukopenia virus | CRFK |
| *Feline chlamydia* | CRFK |
| Canine parvovirus | CRFK |
| Canine distemper | Vero |
| Canine adenovirus type 2 | Vero |
| *Ehrlichia canis* | DH82 |
| Rabies | BHK-21 |
| Eastern equine encephalitis virus | Vero |
| Western equine encephalitis virus | Vero |
| Equine rotavirus | MA104 |
| Equine rhinopneumonitis virus type 1 and 4 | Equine Dermal |
| Equine influenza virus | MDCK |
| Foot and mouth disease virus | BHK-21 |
| Swine parvovirus | ST, PK |
| Swine influenza virus | MDCK |
| *This table was provided by Terry Ng, 2001. Organisms in italics are intracellular parasitic bacteria. | |

## *Biosimilars or Follow-on-Biologics*



**Current sales and estimated patent expiry of selected protein products**

- The terms "Biosimilar" or "Follow-on Biologic" refer to products that are marketed after expiration of patents, which are claimed to have similar properties to existing biologic products. Due to the complexity of biologics, a product can only be made that is similar, but not identical.
- Driving investment in follow-on-biologics is the fact that a number of commercially successful therapeutic proteins will go off patent between 2013-2017, including blockbuster drugs Remicade and Humira
- A difficult question is how much similarity does a biosimilar have to show to its reference product.
- While a biosimilars approval pathway has been established in Europe, US has yet to lay down any guidelines.
- Sandoz was the first company to launch a biosimilar-human growth hormone Omnitrope in both Europe and the United States.

**Approved Biosimilars in the EU**

| Generic Name | mAb type | Manufacturer | Reference product | Launch |
|---|---|---|---|---|
| Abseamed | Recombinant human EPO-α | Medice Arzneimittel Putter (Germany) | Eprex/Erypo (epoetin α Janssen-Cilag, Beerse, Belgium) | 2007 |
| Binocrit | Recombinant human EPO-α | Sandoz (Austria) | Eprex/Erypo (epoetin α Janssen-Cilag, Beerse, Belgium) | 2007 |
| Epoetin alfa Hexal | Recombinant human EPO-α | Hexal Biotech (Germany) | Eprex/Erypo (epoetin α Janssen-Cilag, Beerse, Belgium) | 2007 |
| Retacrit | Erythropoietin zeta | Hospira Enterprises (Netherlands) | Eprex/Erypo (epoetin α Janssen-Cilag, Beerse, Belgium) | 2007 |
| Silapo | Erythropoietin zeta | STADA Arzneimittel (Germany) | Eprex/Erypo (epoetin α Janssen-Cilag, Beerse, Belgium) | 2007 |
| Omnitrope | Somatropin growth hormone | Sandoz (Austria) | Genotropin (Pfizer) | 2006 |
| Valtropin | Somatropin growth hormone | Biopartners (Germany) | Humantrope (Eli Lilly, Netherlands) | 2006 |

**Marketed Biosimilars in India**

| Company | Brand Name | Biosimilar | Launch |
|---|---|---|---|
| Ranbaxy | Ceriton | Epoetin | 2003 |
| Dr Reddy's | Grastim | G-CSF | 2001 |
| | Reditux | MabThera | 2007 |
| Wockhardt | Wosulin | Insulin | 2003 |
| | Wepox | Epoetin | 2001 |
| | Biovac-B | Hepatitis B | 2000 |
| Biocon | Insurgen | Insulin | 2004 |
| | BioMab-EGFR | MabThera | 2006 |
| | Recosulin | Epoetin | 2004 |
| Intas Pharmaceuticals | Epofit, Erykine | Epoetin | 2005 |
| | Neukine | G-CSF | 2004 |
| Shantha Biotechnics | Shanpoietin | Epoetin | 2005 |
| | Shanferon | IFN α 2b | 2002 |
| | Shankinase | Strptokinase | 2004 |
| | Shanvac B | Hepatitis B | 1997 |

**Marketed Biosimilars in China**

| Company | Biosimilars |
|---|---|
| Dragon Pharmaceuticals | Epoetin, filgrastim |
| Dongbao | Insulin, G-CSF |
| Anhui Anke Biotechnology | HGH, interferon alpha |
| Amyotop | G-CSF, IL-11 |
| GeneLeuk Biotech | G-CSF, PEG filgrastim, interferon |
| HangzhouJiuyan Gene | G-CSF, IL-11 |

***Example of Manufacturing Plants***

- Genentech's New Vacaville Facility, California
  - Started construction in 2004, FDA licensure expected 2009
  - Investment: $800 million
  - Eight 25,000-liter bioreactors
  - Production of Herceptin, Avastin and Rituxan
- Bristol Myer Squibb, Devens, Masschusetts
  - Started construction in 2007, validation in 2011
  - Investment: $750 million
  - Six 20,000- liter bioreactors, one purification strain
  - Production of Orencia and other biologics
- Biogen IDEC LSM Facility
  - 245,000 ft2 production
  - Multi-product facility
  - Six 15,000L production reactor capacity

**Dose of some antibody product**

| Product | Disease Indication | Company | Formulation Configuration |
|---|---|---|---|
| Amevive | Psoriasis | Biogen | 7.5mg/0.5ml; 15mg/0.5ml |
| Enbrel | RA | Amgen | 25mg |
| Heceptin | Breast Cancer | Genentech | 440mg/30cc |
| Humira | Rheumatoid arthritis | Abbott, CAT | 40mn (1ml prefilled syringe) |
| Remicade | Crohn's disease, RA | Johnson & Johnson, Centocor | 100 mg/20cc |
| Rituxan | NHL | Genetech/Idec | 100mg/10cc; 500mg/50cc |
| Synagis | Respiratory syncytial virus | MedImmune | 100mg |
| Xolair | Allergic Asthma | Genetech/Tanox/Novatis | 150mn/5cc |

# Alternative Technologies

Mammalian cells are still the main workhorses for biologic production. The other host cells used for biopharmaceutical production include E.coli and Sacchromyces cerevisiae. Alternative production systems include the following:

- Insect cell culture
- Yeast ( Pichia )
- Transgenic animals
- Transgenic plants

## Insect Cell Culture

| Application | Comments |
|---|---|
| Basic research | Hundreds of genes have been expressed using baculovirus. |
| Bioproduction | Possibly 25 or more compounds in clinical trials are being produced using baculovirus expression systems. |
| Gene therapy | A growing number of cell types have been shown to work with BV as the gene-delivery vehicle. |
| Display systems | Demonstrations of the ability to produce large numbers of proteins suggests BV display systems could lead to combinatorial proteomics. |
| Bioreagent production | A number of bioreagent suppliers use BV to make target proteins, viral components and other compounds for the research market. |
| Artificial chromosomes | The large BV genomes suggest use as artificial chromosomes. So far, one patent has been awarded for this application. |
| Biopesticides | There has been an enduring interest in using BV for biopesticides; many attempts have failed. One of the latest is to use a gene for a scorpion toxin against targeted insects. |

## Yeast

Several advances have been made in 'humanizing' the glycosylation characteristics in Pichia systems. Glycofi(Merck) has worked towards a multistep genetic engineering process where they first eliminated non-human glycosylation enzymes and subsequently introduced human glycosylation reactions. They currently report a titer of ~ 1.4g/L. If further developed and scaled up, these engineered yeast strains will facilitate batch to batch consistency by producing uniformly glycosylated products.

| Product | Company | Use | Status |
|---|---|---|---|
| Medway (recombinant human serum albumin) | Mitsubishi Tanabe Pharma Corporation, Osaka, Japan | Blood expander | On the market in Japan |
| Hepatitis B vaccine | Shantha Biotechnics Ltd., India | Hepatitis B | On the market in India |
| Interferon-alpha | Shantha Biotechnics Ltd., India | Hepatitis C/Cancer | On the market in India |
| DX-88 | Dyax Corporation, Cambridge, Mass. | Hereditary angioedema (HAE), a debilitating condition characterized by acute attacks of inflammation. | BLA submitted |
| Recombinant Human Insulin | Biocon, India | Diabetes, all types | On the market in India |
| Recombinant collagen | Fibrogen Inc., South San Francisco | Medical research reagents and dermal filler | On the market |
| Botulism vaccine | USAMRIID/DynPort | Botulism vaccine product | Phase I (U.S.) |
| Anti-thrombolytic | ThromboGenics Ltd. | Thrombosis Tx | Phase II |

## Transgenic Animals

Transgenic organisms for the production of biotherapeutics have been in development for more than a decade. These production systems require low initial capital investment, and have a relatively easy purification process for the glycosylated products. However, so far, only one product has been approved by the FDA- ATryn, produced in transgenic goat milk by GTC Biotherapeutics.

**Animal Species Commonly Used for Expression of Recombinant Proteins in Milk**

| Species | Reproductive Age (months) | Average # of Offspring | Average Yield per Natural Lactation (l) |
|---|---|---|---|
| Mouse | 1 | 10 | 0.0015 |
| Rabbit | 6 | 8 | 1.5 |
| Pig | 8 | 9 | 120 |
| Sheep | 8 | 2 | 400 |
| Cattle | 15 | 1 | 10,000 |

**Transgenic Animal Products Approved or Under Development**

| Species | Company | Product | Status | Comments |
|---|---|---|---|---|
| Goat | GTC Biotherapeutics, MA | ATryn- recombinant human antithrombin-alpha | Approved | Glycosylation patterns differ slightly ( involves N-glycolylneuramic acid- not seen in humans), but was not a regulatory hurdle; Predicted sales of $6-$10 million in 2009. |
| Goat | PharmAthene, MD | Protexia- recombinant human butyrylcholinesterase (BChE) | Development | |
| Rabbit | Pharming, Netherlands | Rhucin-Recombinant human C1 esterase inhibitor | Phase 3 trials | For the treatment of hereditary angiodema. |
| Chickens (eggs) | Origen Therapeutics, Medarex Inc., CA | mAb | Pilot Studies | Functional Mabs produced at 3 mg/egg; some differences in glycosylation; Half life in mouse serum half that of natural antibodies (reduced from 200-100h) |

# Product Quality and Process Robustness

## *Critical Feature of rDNA proteins from mammalian cells*

- Folding and disulfide bond
- Glycosylation
  -N or O - glycosylation
  -Sulfation or phosphorylation of glycans
  -Affect solubility, clearance and biological activities
- Other post-translational modifications
  - Y-carbonxylation
  - lipidation

**Tissue Plasminogen Activator (tPA)1**

- Single polypeptide chain (70 kDa) or proteolytically cleaved at ARG276.
- Multiple N-linked carbohydrates: ASN117 (high mannose), ASN184 (50% complex multiantenary, 50% unoccupied), THR61 (O linked fucose).
- Contains 35 cysteine residues, 17 pairs of disulfide bonds. CYS83 can form a disulfide with other free thiols depending upon the growth medium and buffer composition.
- May form high molecular weight aggregate (complexes with protease inhibitors) and proteolytically cleaved tPA.

**Erythropoietin**

- Contains 40% carbohydrate, only 2 disulfide bonds.
- 3 N-linked ASN (24,38,83), 1 O linked (SER126) glycosylation sites.
- O-linked site not essential for in vitro or in vivo activity.
- Sialic acid residues (average 10 moles/mole Epo) responsible for preserving pharmacokinetic behavior. Muteins lacking 2 or 3 N-linked sites are poorly secreted.
- N linked glycosylation and sialylation is critical to optimal secretion, structure, in vivo potency.

**In Process Structural Alternations to Mammalian Protein Biologics**

| | |
|---|---|
| **Glycoform** | |
| Site occupancy | Possibly caused by stochasticity of glycosylation process |
| Altered sialic acid content | |
| Uncapped galactosyl residue, High mannose | |
| Glycan distribution out of range | |
| **Amino acid alterations in protein** | |
| Mis-incorporation (coding misreading) | Error rate of amino acid incorporation during translation (1/1000) |
| Deamidation (Asparagine) | Most likely occurred in culture fluid, may be affected by process conditions, or even product titer |
| Loss of terminal amino acid<br>• lysine in C-terminus of heavy chain IgG, enzymatic cleavage<br>• cyclization of N terminus glutamine | |
| Glycation (addition of reducing sugar (glucose) to amino acids) | |
| **Protein aggregation** | May be caused by folding in ER or agglomeration in culture or in down stream processing |